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Cloned GABA receptors are maintained in a stable cell line: allosteric and channel properties

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The cloned cDNAs encoding the $\alpha 1$ and $\beta 1$ subunits of the bovine brain GABA_A receptor have been co-transfected, using a dexamethasone-inducible promoter, into cultured hamster ovary cells, with selection to form a stable cell line. The use, alternatively, of a much stronger constitutive promoter led to cell death consequent upon high receptor density. After induction, the cells contained the $\alpha 1$ and $\beta 1$ mRNAs. The expressed receptors showed the high-affinity binding of [³H]muscimol and of the GABA_A receptor channel blocker, t-butylphosphorothionate (TBPS), and the characteristic enhancement of the former by a pregnanolone. Their GABA-activated current was potentiated by the barbiturate, pentobarbitone, was reversibly blocked by bicuculline and picrotoxin, but was not enhanced by benzodiazepines. In mouse spinal cord neurons GABA activates channel openings to at least four conductance states (45, 30, 19 and 12 pS) with the 30 pS state being the most frequently observed (main) state. However, the main state of the $\alpha 1/\beta 1$ GABA_A receptor was the 19 pS state. The enhancement of GABA_A receptor current by barbiturates was due to prolongation of mean channel lifetime, whereas the reduction of GABA_A receptor current by picrotoxin was due to reduction of channel opening frequency and mean channel lifetime. Stable cell lines containing subunit combinations of this receptor should provide a powerful tool for the elucidation of its channel features and control mechanisms.

GABA_A receptors; Expression; Cell lines (stable); Patch-clamping; Single channels

1. Introduction

GABA (γ -aminobutyric acid) operates a gated Cl⁻ channel in the GABA_A receptor of vertebrate brain, one of whose most interesting features is its allosteric regulation at a series of sites which recognize respectively benzodiazepines, barbiturates, certain steroids and convulsants such as TBPS

(t-butylphosphorothionate; reviewed by Barnard, 1988). Further, this receptor has recently been established by DNA cloning approaches to exist in a number of subtypes (Levitan et al., 1988b). The minimum subunit structure appears to contain α and β types of subunit (which themselves exist in multiple subtypes, e.g. $\alpha 1$, $\alpha 2$, etc.) in a form of the receptor that is essentially insensitive to benzodiazepines (Blair et al., 1988; Levitan et al., 1988a), whereas a third type of subunit (γ) can be present in the more usual benzodiazepine-sensitive forms (Pritchett et al., 1989). Transient expression has been achieved of ($\alpha + \beta$) subunits in

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the *Xenopus* oocyte (Schofield et al., 1987; Levitan et al., 1988a,b), and of ($\alpha + \beta$) or ($\alpha + \beta + \gamma$) subunits in cultured mammalian cells (Pritchett et al., 1988; 1989). We have now obtained inducible, stable expression of ($\alpha + \beta$) subunits in a mammalian cell line, and show that these form a stable receptor/channel. The results demonstrate also that the native type of bicuculline, barbiturate, TBPS and steroid allosteric regulatory systems are present within the α and β subunits.

2. Materials and methods

2.1. Plasmid construction and cell transfection

The α and β cDNAs encoding the bovine GABA_A receptor α and β subunits (Schofield et al., 1987) were cloned (using synthetic *Nhe*I linkers) into the polylinker of a plasmid containing the mouse mammary tumour virus (MMTV) promoter, plus an ampicillin-resistance marker and a poly-adenylation acceptor sequence. Each was orientated by restriction analysis, to yield plasmids MMTV- α and MMTV- β . The α and β cDNAs were also cloned into the polylinker of plasmid p-Bex 4 (British Biotechnology, Ltd.) which is similar but contains the human CMV promoter to yield plasmids HCMV- α and HCMV- β . Selection of stable cell lines with either vector is due to the presence, also, of a gpt resistance cassette.

Into the same plasmids (without GABA receptor cDNA inserts) the chloramphenicol transferase (CAT) gene was inserted using an identical method, to yield the plasmids MMTV-CAT and HCMV-CAT. CAT activity was assayed after expression as described by Gorman (1985).

Cultured hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum, 2 mM glutamine, 100 u/ml penicillin-G and streptomycin at 37°C in 5% CO₂. For transfection, exponentially growing cells were trypsinized, seeded at 5×10^5 cells per 9 cm dish and incubated overnight in 10 ml growth medium. Cells were then transfected using the calcium phosphate/BES method as described by Chen and

Okavama (1987). Linearized α and β cDNAs were mixed in a 1:1 ratio; 5 μ g DNA was used per dish. Then 48 h after transfection, the cells were trypsinized and seeded at 1×10^5 cells per dish. For selection, the growth medium was supplemented with xanthine (0.25 mg/ml), mycophenolic acid (5 μ g/ml) and $1 \times$ HT supplement. All medium constituents were from Gibco. Resistant colonies were evident 5-10 days after selection; individual colonies were isolated and grown up in selective growth media.

For RNase A protection analysis two further constructs were produced: a 527 bp *Eco*RI *Sca*I fragment, encoding the first 527 bp of the GABA α subunit (Schofield et al., 1987), was cloned into the polylinker of plasmid p-Gem 4 (Promega Biotech) to yield p-4 α , and a 300 bp *Kpn*I *Eco*RI fragment encoding nucleotides 403-703 of the β 1 subunit was cloned into the polylinker of p-Gem 4Z to yield plasmid p-4Z β 1. Plasmid DNA was prepared by Triton X-100 lysis followed by CsCl fractionation, as described by Maniatis et al. (1982). Prior to transfection the MMTV- α and MMTV- β 1 vectors were linearized by digestion with *Nar*I.

2.2. Introduction and isolation of total PNA

Resistant cells were expanded from stocks maintained in liquid nitrogen in T75 tissue culture flasks to half confluence. Dexamethasone was added to a final concentration of 2 μ M to induce transcription of the MMTV promoter. At 48 h after induction the cells were washed with DMEM before preparation of total RNA using guanidinium isothiocyanate (Chirgwin et al., 1979).

2.3. RNase protection analysis

³²P-labelled RNA antisense probes were produced by in vitro transcription of *Eco*RI linearized p41Z α (utilising the T7 promoter) and of *Kpn*I linearized plasmid p4 β 1 (as described by Treisman (1987)). RNA probes were purified by denaturing gel electrophoresis (Maniatis et al., 1982). The relevant radiolabelled probe (5×10^5 cpm) was then hybridized with 50 μ g total RNA from resistant colonies transfected with the α and

$\beta 1$ GABA receptor cDNAs, or total RNA from untransfected control CHO cells, overnight at 45°C in 80% formamide/100 mM NaCl/80 mM PIPES (pH 6.4)/5 mM EDTA. The reaction mixtures were then treated with RNase A (40 μ g/ml) and RNase T1 (0.5 μ g/ml) in the total volume of 550 μ l of 100 mM EDTA/20 mM Tris-HCl (pH 7.5). Protected fragments were phenol-extracted, ethanol-precipitated, and separated on denaturing gels (Maniatis et al., 1982). The gels were then exposed to Fuji X-ray film at -70°C using an intensifying screen.

2.4. Southern blots

Genomic DNA was isolated from confluent flask cultures of resistant clones, as described by Maniatis et al. (1982). A 10 μ g sample of DNA was digested with *Xba*I, and Southern-blotted from 1% agarose gel onto nitrocellulose. Blots were hybridized with a randomly labelled (Feinberg and Vogelstein, 1983) cDNA (1.8 kb) encoding the entire $\beta 1$ subunit of the GABA_A receptor. Hybridisation was in 50% formamide 5 × SSC (1 × SSC = 0.15 mM NaCl, 15 mM trisodium citrate), 0.5% SDS, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, 0.1% bovine serum albumin, 250 μ g/ml sonicated salmon sperm at 42°C for 2 h, before the addition of radiolabelled $\beta 1$ subunit probe at 10⁶ cpm/ml. The blot was finally washed in 0.1 × SSC at 65% for 1 h.

2.5. Immuno-blots

Crude membranes were prepared from confluent flask-cultures of resistant clones induced for 48 h with dexamethasone prior to harvesting. The cells were homogenized in 4% Triton X-100/100 mM MgCl₂/10 mM Tris-HCl (pH 7.5) in the presence of 10 mM EDTA and EGTA. Crude bovine brain membranes and pure bovine GABA_A receptor (Sigel and Barnard, 1984) served as positive controls, while crude membranes prepared from untransfected CHO cells served as further controls. Extraction into sodium dodecyl sulfate (SDS) sample buffer, precipitation by trichloroacetic acid, SDS gel electrophoresis and Western blotting were all performed as described by Casa-

lotti et al. (1986), using a monoclonal antibody, 1A6, to the GABA_A receptor α subunit, described by Mamalaki et al. (1987).

2.6. Cell culture

Spinal cords were dissected from 12-14 day old murine fetuses and grown in cell culture as described previously (Macdonald et al., 1989a). Cultures were maintained for 2-5 weeks prior to being used in these experiments.

2.7. Electrophysiology; whole cell recording

Experiments were performed using the whole-cell recording technique with a List EPC7 patch clamp amplifier. SCG (superior cervical ganglion) neurones were obtained from E21 rats and grown in culture as previously described (Smart, 1987). The CHO cells and SCG neurones were continuously superfused in the culture dish at 28°C with a HEPES-Krebs solution of the composition (mM): NaCl 140, KCl 4.7, MgCl₂ 1.2, CaCl₂ 2.5, glucose 11, HEPES 5, buffered to pH 7.4. Electrodes were fabricated from borosilicate glass and heat-polished to a final diameter of 0.5-1.5 μ m. The recording pipette solution contained either (mM): KCl 30, KAsp 120, MgCl₂ 1, EGTA 0.5, CaCl₂ 0.28, HEPES 5, buffered to pH 7.0 with 1 M NaOH (final concentration 3 mM); or KCl 150, MgCl₂ 1, EGTA 0.5, CaCl₂ 0.28, HEPES 5, buffered to pH 7.4 with 1 M NaOH. Data were recorded on a Brush-Gould ink-jet pen recorder. GABA was either bath-applied or applied from an adjacent patch pipette filled with 50-500 μ M GABA and ejected using N₂ pressure. Healthy CHO cells had resting potentials varying from 32 to -58 mV with a linear I/V relationship, while SCG neurones had resting potentials of -48 to -72 mV and spike amplitudes of > 100 mV.

2.8. Electrophysiology; single channel recording

The medium used to grow and maintain the cultures was exchanged for an extracellular salt solution which consisted of the following, in mM: 142 NaCl, 8.1 KCl, 1 CaCl₂, 6 MgCl₂, 10 glucose, 10 Na-HEPES (pH 7.4). The intrapipette solution

contained, in mM: 153 KCl, 1 MgCl₂, 10 K-HEPES, 5 EGTA (pH 7.38). This combination of extracellular and intrapipette solutions resulted in a chloride equilibrium potential (E_{Cl}) of about 0 mV and a potassium equilibrium potential (E_K) of -75 mV across the patch membrane. All experiments were performed at room temperature (20-23°C).

GABA was diluted with extracellular solution from a 10 mM stock solution to a final concentration of 5 μ M on the day of the experiment. All drugs were obtained from Sigma Chemical Co., (St. Louis, MO, U.S.A.). GABA or GABA with a drug was applied to the patch membrane using

pressure ejection micropipettes which were moved to within 50 μ m of patches only during the time of drug application.

Recording techniques were similar to those previously described (Hamill et al., 1981; Macdonald et al., 1989). Excised outside-out patches were obtained using a List model L/M EPC-7 patch clamp amplifier and were simultaneously recorded on a video cassette recording system (VCR) (SONY SL-2700) via a digital audio processor (SONY PCM-501 ES, modified to 0-20 kHz, 14-bit resolution, 44 kHz sampling frequency) and a Brush-Gould ink-jet pen recorder. At a later time, the data were played back from the VCR and digitized (20 kHz, 14 bit) with a 2 kHz (3 dB) 8-pole Bessel filter interspersed. Current amplitudes and durations were determined by computer using software previously described (Macdonald et al., 1989). Openings and closings were detected using the 50% threshold crossing method and were accepted as valid events if their durations were greater than twice the system dead time (dead time = 70 μ s). Measured open times will generally be longer than 'true' open times due to undetected openings. Throughout the text the term 'apparent open time' will refer to measured time that has not been corrected for unobserved transitions (McManus et al., 1987).

3. Results

3.1. Transfection and comparison of expression with two vector types

CHO cells were transfected with plasmids containing (separately) the $\alpha 1$ and $\beta 1$ subunit cDNAs of the bovine GABA_A receptor. These were driven either by a human cytomegalovirus promoter (HCMV) or by a mouse mammary tumour virus promoter (MMTV), both with selection by gpt resistance. It was found that the MMTV ($\alpha 1 + \beta 1$)-transfected cells could be selected through many passages with good yield throughout, whereas the HCMV ($\alpha 1 + \beta 1$)-transfected cells died out in the first growth cycle after transfection (table 1). The latter behavior was attributable to the presence of the GABA_A receptor in this

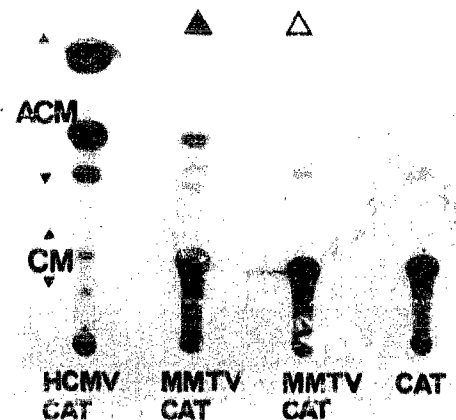


Fig. 1. Comparison of the strengths of the HCMV and MMTV promoters in CHO cells. CHO cells were transfected with the indicated plasmids (see Materials and methods). CAT was derived from HCMV-CAT by deletion of the HCMV promoter. Cells were re-supplied 24 h after transfection with either normal growth medium (HCMV-CAT and CAT), or medium supplemented with 10^{-6} M dexamethasone (\blacktriangle), or medium supplemented only with ethanol (dexamethasone solvent) to 0.05% (\triangle). Cells were harvested 48 h later, lysed by repeated cycles of freezing and thawing, and the induced CAT activity was assayed. ACM, acylated chloramphenicol; CM, chloramphenicol. Quantitation showed that the percentage conversion to all products was 100 times greater for HCMV than for MMTV (induced), and MMTV was a further five- to 10-fold weaker when not induced.

TABLE 1

Comparative yields of *gpt*-resistant CHO colonies after transfection. Exponentially growing cells (5×10^5) were co-transfected with the pairs of plasmids shown, or with HCMV-CAT alone, which contains a chloramphenicol acetyltransferase gene in place of a GABA receptor subunit cDNA. After transfection the number of resistant colonies was determined after 7 days in the selection medium.

Plasmids	Number of resistant colonies per 10^6 cells
MMTV- $\alpha 1$ + MMTV- β	$> 10^3$
HCMV- $\alpha 1$ + HCMV- β	~ 1
HCMV-CAT	$> 10^3$

HCMV-driven expression, since when the receptor was replaced by the CAT gene the toxicity disappeared, and the same was true when other non-re-

ceptor DNAs were expressed therein. Further, the HCMV-transfected cells with $\alpha 1 + \beta 1$, containing only CAT as the exogenous gene, constantly expressed a high level of CAT activity (fig. 1). Likewise, use of the SV40-early promoter led to the same unstable effect as the HCMV promoter (data not shown). In contrast, the constant growth of the MMTV-CAT-transfected cells was accompanied by little expression of CAT; this could be increased five- to 10-fold by the induction with dexamethasone.

3.2. Characterisation of the *gpt*-resistant clones

The cells co-transfected with MMTV- $\alpha 1$ and MMTV- $\beta 1$ and then *gpt*-selected were shown to

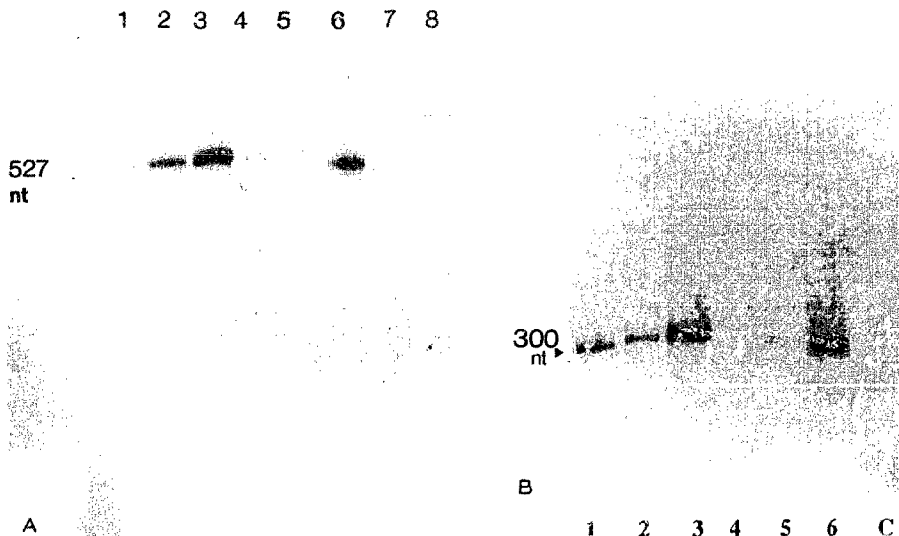


Fig. 2. RNase A protection analysis. Total RNA was harvested from 6 separate cell lines transfected with both the $\alpha 1$ and $\beta 1$ subunits of the GABA receptor subunits under the control of the inducible MMTV promoter, and from control CHO cells. The RNA was then hybridized with the $\alpha 1$ subunit antisense probe (A) or with the $\beta 1$ subunit antisense probe (B). RNase-resistant fragments were separated on a 6% acrylamide/7 M urea gel (Zinn et al., 1983). Lanes 1-5 (A and B): RNA from different cell lines; lane 7 (A) and C (B) control (untransfected) cells. Undigested probe was also included in A (lane 8). End-labelled fragments of a *Msp*I digest of pBR322 served as size markers (not shown). Gels were exposed to Fuji film at -70°C for 7 days. The same range of intensity of expression as seen here was found in other cell lines in which $\alpha 1$ and $\beta 1$ alone had been stably introduced, when the respective probes were used alone (not illustrated).

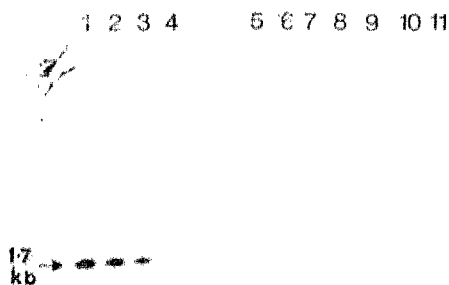


Fig. 3. Southern blot of DNA isolated from ($\alpha 1 + \beta 1$) cell lines, hybridized with α/β subunit probe. 10 μ g of genomic DNA from 6 cell lines co-transfected with the GABA receptor $\alpha 1$ and $\beta 1$ subunit cDNAs (lanes 5-10) or control CHO cells (lane 11) or (lanes 1-4) 48, 36, 24 and 12 μ g of plasmid MMTV- β were digested with *Nhe*I, electrophoresed on a 1% agarose gel and Southern blotted. The probe used was the entire $\beta 1$ subunit cDNA. Exposure was to Fuji film at -70°C using an intensifying screen for 24 h. In the marker lane (extreme left) a 1.6-kb marker band is visible.

have stably integrated the $\alpha 1$ and the $\beta 1$ subunit cDNAs. This is shown by Southern blotting in fig. 2 for the $\beta 1$ cDNA, with standard quantities of the insert alone for comparison. The results show that from one to five copies have been integrated per cell. Identical results were obtained with the $\alpha 1$ insert in the same cells (data not shown).

The subunit transcripts produced in these cells (after induction for 48 h) were recognized by RNase protection with the corresponding probes (fig. 3). This showed that all of the cell lines produced contain the $\alpha 1$ and $\beta 1$ mRNAs together. The amounts varied considerably and at random between the cell lines, depending upon the position of genomic integration of the foreign DNA. However, the α and β DNAs always were co-incorporated to the same order of magnitude, and the same range of incorporation between stable lines was shown when $\alpha 1$ or $\beta 1$ were transfected alone (not shown). Since the $\alpha 1$ and $\beta 1$ probes were equally labelled and their sizes were fairly

similar, the results showed that cell lines 3 and 6 had the highest levels of both $\alpha 1$ and $\beta 1$ mRNA and these were chosen for long-term use.

3.3. Reactions with anti-GABA receptor monoclonal antibody

The production of subunit polypeptide was monitored by immunoblotting. This was only possible for the $\alpha 1$ subunit, since no antibody specific for the $\beta 1$ subunit has yet been obtained.

Using a monoclonal antibody, 1A6, directed against α subunits (Mamalaki et al., 1987), it was shown (fig. 4) that the $\alpha 1$ polypeptide was recognized and was present in ($\alpha 1 + \beta 1$) and in $\alpha 1$ -alone transfected cells. The $\beta 1$ subunit was not recognized, either in ($\alpha 1 + \beta 1$) or in $\beta 1$ -alone transfected cells. As noted previously (Mamalaki et al., 1987), a band in the β region was recognized by 1A6 in the receptor from native brain membranes

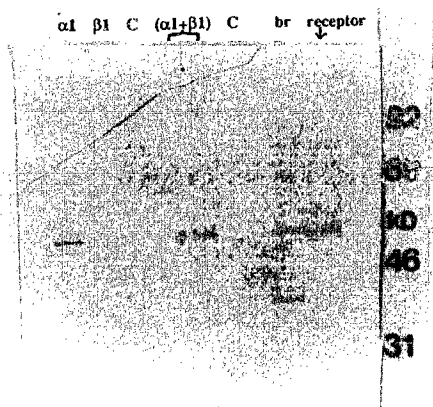


Fig. 4. Immuno-blot after ($\alpha 1 + \beta 1$) subunit expression in CHO cells. Samples (100 μ g protein) from cells transfected with MMTV- $\alpha 1$ (lane 1), MMTV- $\beta 1$ (lane 2), MMTV- $\alpha 1$ plus MMTV- $\beta 1$ (lanes 4 and 5) were electrophoresed and immunoblotted (see Materials and methods). Also included in the blot were crude bovine brain membranes (br; 150 μ g of protein), purified GABA_A receptor protein (100 ng; arrow) and membranes from untransfected cells (control, C). The positions of molecular weight standards (BRL Ltd) are indicated. The band at ~ 40 kD from br is known to be a degradation product of the $\alpha 1$ subunit (Mamalaki et al., 1987).

TABLE 2

Binding sites in cell membranes transfected with ($\alpha 1 + \beta 1$) subunits. Ligands (Amersham) were at 25.3 Ci mmol⁻¹ for muscimol or 87 Ci mmol⁻¹ for TBPS, each at 40 nM or saturating concentration. Nonspecific binding was measured in the presence of 1 mM GABA (muscimol), 10 μ M picrotoxin (TBPS) or 3 μ M clonazepam (flunitrazepam). Membranes prepared from cells and assayed as in Casalotti et al. (1986). Values are means \pm S.E. of triplicates in three independent determinations.

Ligand	fmol/mg protein	fmol/10 ⁶ cells	Binding sites/cell
[³ H]Muscimol	15.6 \pm 3.7	0.26	156
[³⁵ S]TBPS	13.7 \pm 2.0	0.23	138
[³ H]Flunitrazepam	None	None	None

(fig. 4), but it could be seen that this is not the $\beta 1$ subunit and could be attributed to the $\alpha 3$ subunit, present in the receptor purified from bovine cortex and migrating in the $\beta 1$ region (Stephenson et al., 1989).

3.4. Ligand binding of the expressed receptors

The membranes of the ($\alpha 1 + \beta 1$) transfected, dexamethasone-induced cells bound [³H]muscimol and [³⁵S]TBPS in the nanomolar range (table 2). The amounts were low, corresponding to only 100-300 binding sites per cell, but these levels were reproducible and significant.

The GABA_A receptor in native neurones is sensitive to certain steroids of the progesterone/alpha xalone/corticosterone series (Peters et al., 1988), and this can be monitored by their enhancement of the binding of GABA or muscimol (Harrison and Simmonds, 1984). This effect was present, to the same degree, when the ($\alpha 1 + \beta 1$)

TABLE 3

Enhancement of [³H]muscimol binding by 5 β -pregnan-3 α -ol-20-one (100 μ M) in cell membranes.

Sample	[³ H]muscimol (40 nM) binding (% of control)
Bovine cortex membranes	170%
($\alpha 1 + \beta 1$)-Transfected cell membranes	160%
Untransfected cell membranes	None

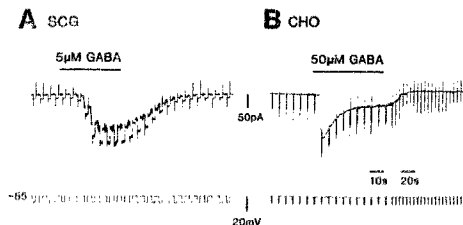


Fig. 5. Response to GABA of ($\alpha 1 + \beta 1$)-transfected CHO cells and of SCG neurones. (A) A sympathetic neuron was whole-cell voltage-clamped at -55 mV and subjected to 10 mV, hyperpolarizing commands for 500 ms every 5 s. Bath application of 5 μ M GABA evoked an inward current coupled with a clear increase in membrane conductance and membrane current noise. The response peak was well maintained, with only slight fading during application of the agonist. Note that the chart recorder speed was intermittently increased during each voltage command. (B) GABA responses were recorded from CHO cells at -55 mV (hyperpolarizing voltage steps of 10 mV, for 500 ms) following bath application of 50 μ M GABA, which was chosen to produce a 'matched-current' response compared to (A). The CHO-cell response profile differed from that observed in cultured SCG neurones by fading quite rapidly during exposure to GABA, which may be representative of a more overt desensitization process in the expressed GABA receptors residing in the CHO cells. In addition, the conductance increase was considerably smaller in the latter with virtually no discernable membrane current noise. Note the difference in the concentrations of GABA required for CHO and SCG cells to produce matched GABA responses.

transfected cells were tested with one of the steroids involved (table 3).

3.5. Expressed receptor channels

3.5.1. Occurrence and density of receptors

After the dexamethasone-induction of MMTV-($\alpha 1 + \beta 1$) transfected cells, a maximal response to applied GABA could be detected within 48 h upon recording the evoked inward currents (fig. 5). The level of response seen was not changed after numerous passages of the positive cells, so that this cell line was stable. In contrast, the HCMV-($\alpha 1 + \beta 1$) transfected cells initially gave greater currents, but these were very variable between cells and all disappeared after the first growth cycle.

With the MMTV-($\alpha 1 + \beta 1$) transfected cells after induction, the density of receptors, as esti-

mated by the size of the GABA-evoked membrane currents, varied considerably around the mean illustrated in fig. 5. Bath application of 50 μM GABA produced a mean inward current of 81 pA \pm 14.5 pA (standard error from 12 cells obtained from two batches of CHO cells separately induced by dexamethasone). Generally, bath concentrations of GABA required to activate these receptors were about 10 times those required to activate the GABA_A receptors on dissociated mammalian neurones (fig. 5). The relative receptor densities were also apparent when puffing GABA over the CHO cells, which resulted in a small response with a slow onset, despite the pipette being closer than one cell diameter away from the recording site. The peak current obtained from CHO cells (at -55 mV) with GABA applied with 88 kPa pressure was again about 10-fold lower than that obtained from SCC neurones with GABA applied similarly (at 70 kPa) (data not shown).

3.5.2. Reversal potential and pharmacology

The reversal potential for the GABA response followed the Cl⁻ reversal potential closely. For example, when using the aspartate/low Cl⁻ medium in the pipette (see Materials and methods) E_{GABA} was -38 mV ($E_{\text{Cl}} = -40$ mV), and when using the Cl⁻-based medium, E_{GABA} was -4 mV ($E_{\text{Cl}} = 0$ mV).

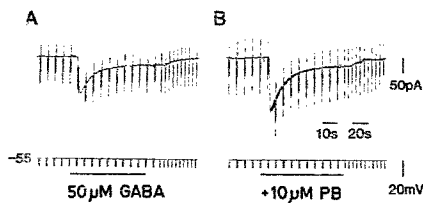


Fig. 6. GABA responses were enhanced by pentobarbitone. The ($\alpha 1 + \beta 1$)-transfected cells were whole-cell voltage-clamped at -55 mV following expression of GABA_A receptors, and subjected to 10 mV hyperpolarizing voltage command steps (lower trace). (A) Bath application of 50 μM GABA produced an inward current (top trace) and a conductance increase which rapidly faded on continued exposure to the agonist. (B) In the presence of 10 μM pentobarbitone (PB), the peak GABA response was clearly enhanced, together with the membrane current noise. This effect of PB was readily reversible on washing.

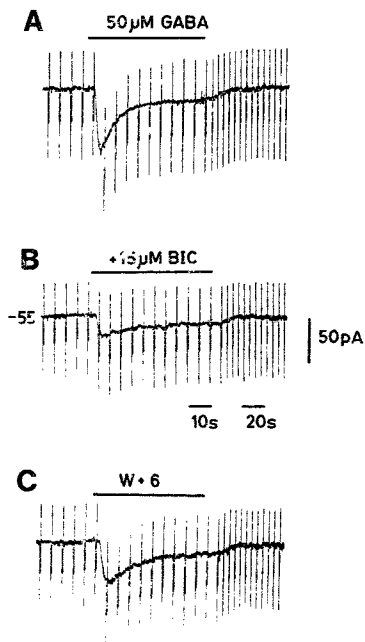


Fig. 7. GABA_A receptors expressed on ($\alpha 1 + \beta 1$)-transfected CHO cells were sensitive to blockade by bicuculline. (A) Control inward GABA currents were routinely produced under whole-cell voltage clamp by the bath application of GABA (50–200 μM). (B) The presence of 15 μM bicuculline for 3 min strongly inhibited the GABA response. (C) Reversal of the bicuculline blockade readily occurred on washing for 6 min.

Enhancement by pentobarbitone (10–50 μM ; $n = 4$ cells) was seen with increases in the range of 50–110% (fig. 6). Bicuculline methobromide also inhibited the GABA response but was less potent than with the SCG neurones (fig. 7). At least 15 μM bicuculline was required to produce a reasonable block of the GABA response; in comparison, concentrations < 10 μM were sufficient to completely abolish the neuronal GABA response on cultured sympathetic neurones. Lastly, when GABA was applied at 10–100 μM , there was no enhancement detectable by the benzodiazepines, flurazepam (5–10 μM) or chlorazepate (5–10 μM) ($n = 8$ cells each).

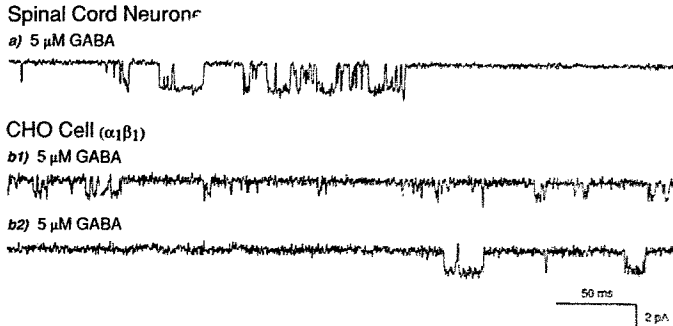


Fig. 8. Single channel Cl^- currents were evoked by GABA in outside-out patches from spinal cord neurones and α 1/ β 1 CHO cells. Patches were voltage clamped at -75 mV. (a) In patches from spinal cord neurones, GABA ($5 \mu\text{M}$)-evoked channel openings were composed predominantly of the 27 pS main conductance state. (b1) A typical response to GABA ($5 \mu\text{M}$) in a patch from an α 1/ β 1 CHO cell. The openings were briefer than in spinal cord neurones, and the predominant conductance in these patches was 19 pS. (b2) An example of the 27 pS conductance state obtained in a patch from an α 1/ β 1 CHO cell. Time and current calibration bars at the lower right apply throughout.

3.5.3. Single channel recordings

GABA_A receptor channels recorded from mouse spinal cord neurones opened to a main conductance state of 27 pS, although occasional 19 and 11 pS openings were recorded (Macdonald et al., 1989), and the channels opened in bursts of openings (fig. 8a). In contrast α 1/ β 1 GABA_A receptor channels recorded from CHO cells opened

to a main conductance state of 19 pS (86% of total current; fig 8b1) with few openings to the 11 pS (4% of total current) and 27 pS (9% of total current) conductance states (fig. 8b2). The channels opened singly and in relatively brief bursts (9313 openings recorded from 20 patches). Openings of the α 1/ β 1 GABA_A receptor channels were briefer than openings of mouse spinal cord

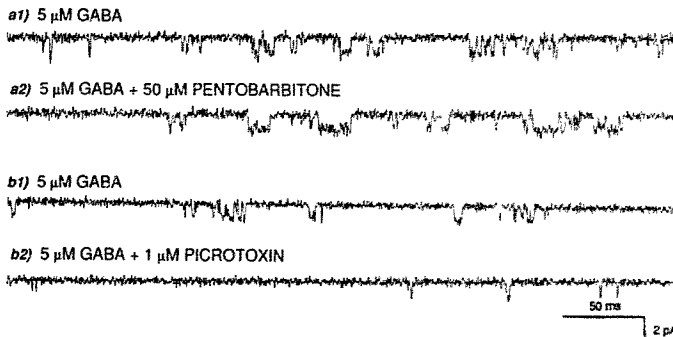


Fig. 9. Pentobarbitone and picrotoxin altered single channel chloride currents evoked by GABA in outside-out patches from α 1/ β 1 CHO cells. (a1 and b1) Responses to $5 \mu\text{M}$ GABA in two different patches before the application of pentobarbitone or picrotoxin, respectively. GABA-evoked openings were predominantly to the 19 pS conductance state. (a2) In the presence of $50 \mu\text{M}$ pentobarbitone, the duration of the openings was prolonged. (b2) Picrotoxin ($1 \mu\text{M}$) reduced both the frequency of channel opening and the duration of the openings. Time and calibration bars at the lower right apply throughout.

GABA_A receptor channels. Mean apparent open times of the $\alpha 1/\beta 1$ and mouse spinal cord GABA_A receptor channels (5 μ M GABA) were 1.7 and 6.0 ms, respectively.

The $\alpha 1/\beta 1$ GABA_A receptor channel current was regulated by barbiturates and by picrotoxin (fig. 9), but was not enhanced^d by the benzodiazepine, diazepam (100 nM; not illustrated). Pentobarbitone (50 μ M) prolonged apparent mean open time to 2.8 ms (an increase of 67%) (fig. 9a2). The increase in open time was accompanied by a decrease in apparent opening frequency from 12.3 to 6.1 openings per s (5868 openings recorded from nine patches). In contrast, picrotoxin (1 μ M) decreased apparent mean open time to 1.4 ms (a reduction of 19%) and decreased apparent opening frequency to 2.2 openings per s (fig. 9b2; 592 openings recorded from three patches).

4. Discussion

A stable cell line has been created carrying α and β subunits of the GABA_A receptor. Only their transient expression has been achieved previously (lasting only 1 day), and an obstacle to deriving stable cell lines carrying this receptor has been identified here: when strong promoters were used with these cDNAs so that the receptors were continuously expressed at an initial high density, the cells did not survive, perhaps due to spontaneous activation of ionic fluxes. Only by the use of an inducible promoter and a low level of induced expression could a cell line carrying this receptor be maintained permanently.

The expressed receptors show the expected binding of muscimol and of TBPS. Their channels show the normal potentiation by a barbiturate and (at the ligand binding level, at least) by a steroid. They are likewise sensitive to bicuculline and picrotoxin.

Potentiation of the GABA-evoked response by benzodiazepines is either absent or too transient to measure in this system. This is to be expected, since it is now known that a γ subunit is required to stabilize this response (Pritchett et al., 1989).

The number of binding sites per cell, in the range 100-300, is low, as is expected from the

known low efficiency of integration of steroid regulated DNAs. Nevertheless it is sufficient for electrophysiological analyses to be made. Further, in recent recloning of such cells this number has risen to about five times that level, which is adequate for binding studies. Calculation based upon the single channel conductance shows that the observed mean whole-cell current for the original population at saturating GABA concentration corresponds to about 300 receptor channels per cell, which is not less than the number estimated from the binding data (table 2); hence, many of the expressed membrane receptors are in the plasma membrane and form functional channels. We routinely observed that in order to produce similar size GABA responses on CHO cells compared to those induced on primary neurones, required approximately ten times more GABA. This apparent disparity in GABA concentration could be due to the exclusion of other essential subunits (e.g., γ , Pritchett et al., 1989) or more likely, reflects the smaller number of receptors expressed in CHO cells. A further contributory factor was the smaller mean conductance state (19sp) for GABA channels observed in CHO cells compared to 27-30 pS observed as the most frequent conductance states in primary neurones.

The expressed receptors have some properties which are similar to those present on mouse spinal cord neurones. The expressed receptor channels were subject to allosteric regulations by barbiturates and picrotoxin. The expressed receptor channel openings were prolonged by pentobarbitone (Study and Barker, 1981; Macdonald et al., 1990; Twyman et al., 1989a,b) and shortened by picrotoxin (Twyman et al., 1989a). However, the expressed receptor channel current was not increased by benzodiazepines (Pritchett et al., 1988). These results are consistent with the observation that barbiturate and picrotoxin regulatory sites are present on α and β subunits but that the γ subunit is required for full expression of benzodiazepine sensitivity (Pritchett et al., 1989).

The single channel properties of the expressed channels were similar to those of mouse spinal cord neurones in the openings of 12, 19 and 27 pS channels which were recorded (Bormann et al., 1987; Macdonald et al., 1989). However, the ex-

pressed channels differed substantially in the relative proportion of openings recorded. In receptors from spina¹ cord neurones, the main conductance state was the 27-30 pS state while in the expressed receptors the predominant state was a 'subconductance state', the 19 pS state. This observation might suggest the possibility that the main state is dependent on the presence of all subunits and that the different subconductance states may be due to the presence of receptors composed of a subset of subunits; however, when the same α 1 and β 1 subunits were previously incorporated into the *Xenopus* oocyte (Levitan et al., 1988b), both in the 18 pS and 28 pS states occurred quite frequently. This could reflect differences in the two expressions systems used, but other criteria, including the pharmacology, appears very similar between GABA_A receptors comprising α 1 and β 1 subunits expressed in *Xenopus* oocytes and CHO cells. A further possibility is that the expressed GABA receptor may exhibit a time-dependent kinetic heterogeneity, allowing the channel to adopt various different conductance states at different times. This phenomenon has recently been reported for a 'native' GABA-operated ion channel in sympathetic neurones (Newland et al., unpublished).

The methodology used here can now be used to create cell lines stably expressing various combinations of the subtypes of GABA_A receptor subunits (unpublished results). The only comparable multisubunit receptor cell line hitherto known is that of the *Torpedo* ACh receptor (Claudio et al., 1987) which was constructed in a fibroblast line, but its long-term stability has not been described. Such stable receptor-bearing lines offer a new resource for pharmacology and electrophysiology. Their reproducibility, accessibility and the ease of patch-clamping are some of the obvious technical advantages. Drug screening with various ligand types (GABA mimetics, benzodiazepines, steroids, etc.) could be readily automated using such cultured cells. Single channel analyses are facilitated by the homogeneous population. However, the greatest benefit of this type of cell line will be, we believe, the ability to isolate the effects due to particular subunits or subtypes of the receptor and to analyze their relationships to the single channel parameters (e.g. subconductance states, desensiti-

sation rates, gating kinetics, etc.). Mutagenesis of sites related to ligand binding and to channel function will acquire a new dimension when it becomes applied to such subtype-specific permanent reservoirs of receptors.

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